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THE ACTION OF THE ANTIPERNICIOUS ANEMIA PRINCIPLE ON THE BLOOD PICTURE OF OPOSSUM POUCH-YOUNG AND RAT EMBRYOS

A PART OF A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY
1941

By
JULES H. LAST

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THE ACTION OF THE ANTIPERNICIOUS ANEMIA PRINCIPLE ON THE BLOOD PICTURE OF OPOSSUM POUCH-YOUNG AND RAT EMBRYOS.*

By Jules H. Last

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The only method of measuring potency of liver preparations is the clinical assay on "suitable," uncomplicated pernicious anemia patients in a state of relapse. The disadvantages in having to depend on the fortuitous appearance of an ever-decreasing number of satisfactory pernicious anemia cases at certain hospitals and clinics for the quantitative assay of a drug are immediately apparent. The present U.S.P. unit of potency for liver preparations is defined on the basis of the remission of the blood picture in "suitable" patients. The unit is vague and unsatisfactory.

Space does not permit a critical discussion of the numerous, biologic tests proposed, but with few unconfirmed exceptions[4,5,9] none would serve as a valid indicator for the potency of liver preparations. Of special interest have been the reports that antipernicious anemia (A-P-A) principle accelerates the maturation of embryonic blood elements. In 1880, Ehrlich pointed out that the morphologic identity of the erythroid cells of the primitive, prehepatic generation of red cells in the embryo and the megaloblastic generation of red cells characteristic of untreated pernicious anemia. He postulated erythropoiesis in pernicious anemia to be an embryonal reversion. At present it is believed that the red cells of the primitive generation in the embryo though morphologically similar are not identical with the megaloblastic generation of pernicious anemia.[6a] The possibility that embryonic and megaloblastic erythropoiesis might respond in a similar manner to liver therapy has stimulated interest in the fetal approach to the bio-assay problem.[15]

In 1928, it was reported that blood island maturation in early chick blastoderms was accelerated by the addition *in vitro* of liver preparations.[3] This work could not be confirmed.[7] The injection of liver extracts into the unincubated egg does not change the blood picture of the developing chick embryo before and during the anlage

* Aided by grant from Armour Research Fund of the University of Chicago.

of the bone marrow.[10] The blood picture in the rabbit fetus has been reported unaffected by the A-P-A principle.[16] It has recently been reported that the injection of anti-anemia principles does alter the blood picture of opossum pouch-young.[12a,b] Extensive studies have been reported on the response of the new-born rat whose mother was treated with anti-anemia substances. Accelerated maturation has been observed[1,11,13] but has not been confirmed.[2,8,14] Recent observations of accelerated maturation of primitive erythropoiesis in the yolk sac of the 11-day rat fetus subsequent to liver and ventriculin therapy in the pregnant rat emphasize the necessity of studying the prehepatic, "megaloblastic" generation of cells.[6b,c]

The positive findings in the opossum pouch-young and 11-day rat has prompted further study of the embryo with the view of developing a method of bio-assay for the antipernicious anemia principle.

Methods. United States Bureau of Standards hemocytometers and Trenner automatic red cell pipettes were used for red blood cell counts. Hayem's solution was the standard diluent. Three-tenths per cent brilliant cresyl blue in isotonic salt solution was used as the diluent and stain for reticulocyte counts. The Newcomer method was used for hemoglobin determinations. Van Allen tubes were used for hematocrit determinations with heparin as the anticoagulant. All dry blood smears were stained with May-Grünwald-Giemsa stain.

In the opossum work, 45-day-old pouch-young, about 65 mm. long (snout-rump length), were injected subcutaneously with daily doses of 3 units of a potent liver preparation, for 7 to 9 days.* The young remained attached to the maternal nipple throughout the injection period. Controls consisted of litter mates either uninjected or receiving an inactive liver preparation (70% alcohol insoluble fraction).† Due to the size of the animals it was necessary to decapitate the young to obtain blood samples for red cell counts and hematocrit determinations. The mother opossums were maintained on a diet of raw meat, fox chow pellets, and water.

In the rat work, young adult female rats from our stock colony (mixed strain) were mated and on the twentieth, fifteenth and eleventh day of pregnancy the fetuses were removed under ether anesthesia with the amnion, yolk sac, and placental button intact. Decapitation of 20-day fetuses was employed to obtain blood samples. Mean corpuscular volumes of the red cells were calculated from red cell counts and hematocrit readings. Blood from the 15-day fetuses was obtained by puncturing the vitelline vessels lying on the yolk sac. The circulating blood of the 15-day fetus is comprised predominantly of erythroblasts, normoblasts, non-nucleated reticulocytes and erythrocytes of the prehepatic generation. Therefore, accelerated maturation of primitive erythropoiesis reported in the 11-day fetus should appear in a study of the blood of the 15-day fetus. Since the percentage of non-nucleated cells rises rapidly between the fourteenth and eighteenth days of fetal life, a significant increase above the control percentage value at 15 days represents accelerated maturation of primitive erythropoiesis. One sample of blood was smeared, dried, and stained as described above. Supravitally stained wet preparations were also made by mixing another drop of blood with 0.18% brilliant cresyl blue in isotonic salt solution. One thousand cells per fetus were counted, 500 on a wet preparation and 500 on a dry smear. The choice of 4 fetuses yielded a

* Generously contributed by Dr. Guy Clark of Lederle Laboratories.
† Obtained through the kindness of Armour Laboratories.

representative sample of a litter. The weight of each fetus was recorded, since the body weight was considered the most accurate gross measure of fetal development in the rat.

The small size of the 11-day embryos required the use of a binocular dissecting microscope to free them from their membranous investments. Blood smears were made from heart blood and stained in the usual manner. The circulating red blood cells of the 11-day rat fetus are, for the most part, immature basophilic cells of the primitive generation. As maturation and mitosis occur intravascularly, the cytoplasm becomes polychromatophilic and hemoglobiniferous. Concurrently there is a marked decrease in nuclear size with condensation of nuclear chromatin. A significant decrease in nuclear diameter would be indicative of accelerated maturation of primitive erythropoiesis. Nuclear diameters were measured by means of a camera lucida. Again, 4 embryos per litter were used and 50 cells per embryo were measured.

In the 20-day embryo group, the liver-treated animals received 7.5 u. on the first day of pregnancy, 7.5 to 15 u. on the ninth, tenth or fourteenth day, and a 1.5-u. daily maintenance dose. The rats received a total of 41.5 to 49 U.S.P. units during the 20-day period of pregnancy. The mother rats in the 15-day group were usually given 3.5 to 7.5 u. on the sixth day of pregnancy and maintained on 1.5 u. daily through the fourteenth day. Rats on a 31-u. level received 7.5 u. on Days 6, 7, and 8, with subsequent 1.5-u. maintenance dose daily. Total doses varied from 9 to 31 U.S.P. units per rat. In the 11-day group, the rats received 12 to 75 u. during the eighth, ninth, and tenth days of pregnancy. In this group pregnant rats were also fed fox chow checquer diets containing 2.5% Ventriculin by weight. This dosage has been reported as optimal for accelerating maturation of the primitive generation in the 11-day embryo. All pregnant rats were treated with potent anti-anemia preparations before, during, and after the anlage of the mesoderm which gives rise to the blood-forming tissues in the embryo. With the exception of the 11-day group which was fed a fox chow checquer diet, the 20- and 15-day groups were fed a stock ration of corn meal, powdered milk, raw meat, bone meal and fresh water.

Results. The blood values of 27 opossum pouch-young are listed in Table 1. The mean corpuscular volumes of the red cells compare favorably in the animals receiving potent liver therapy, inactive liver preparations, and no injections. There is also no appreciable change in the red cell count. The series of animals is of necessity small, due to the difficulties in obtaining opossums from the South and to the fact that the animals breed only in the spring of the year. Contrary to positive reports, no accelerated maturation of the blood picture as reflected by a significant decrease of the mean corpuscular volume of the erythrocytes was observed in the pouch-young receiving liver therapy.

TABLE 1.—THE RESPONSE OF OPOSSUM EMBRYOS TO LIVER THERAPY.

Therapy.	No. of embryos.	Length* (mm.).	Weight (gm.).	R.B.C.†	Hematocrit.‡	M.C.V.§
Controls (initially removed)	6	64	8.2	2.55	30.1	118.7
Controls (uninjected)	2	73	...	3.25	35.2	109.2
Liver extract (inactive)¶	6	75	12.1	2.49	31.5	126.4
Liver extract (Lederle)	13	76	12.4	2.70	30.8	112.3

* Snout-rump length (mean).
† Erythrocytes in millions per $mm.^3$ (mean).
‡ Per cent packed erythrocytes (mean).
§ Mean corpuscular volume in cubic micra (mean).
¶ 70% alcohol insoluble fraction.

The lack of a response to the A-P-A factor in the normal 20-day rat fetus is illustrated in the summary of 46 fetuses in Table 2. The red cell counts and mean corpuscular volumes of the treated animals compare favorably with the uninjected controls.

TABLE 2.—EFFECT OF LIVER EXTRACT ON THE BLOOD PICTURE OF 20-DAY RAT EMBRYOS.

			R.B.C.		Hematocrit.		M.C.V.	
Therapy.	Litters.	Fetuses.	Mill.*	σ.	%.†	σ.	μ^3.‡	σ.
Controls (uninjected) .	4	22	1.95	0.73	36.9	4.9	189.0	18.4
Lederle liver extract, 41.5–49 U.S.P. units	5	23	2.12	0.25	38.5	3.1	185.0	11.5

* Erythrocytes in millions per mm.^{3} (mean).
† Per cent packed erythrocytes (mean).
‡ Mean corpuscular volume in cubic micra.

In the large series of 15-day rat embryos studied, considerable variation in the development of embryos was observed. Figure 1 illustrates the per cent non-nucleated cell values as plotted against fetal weight.

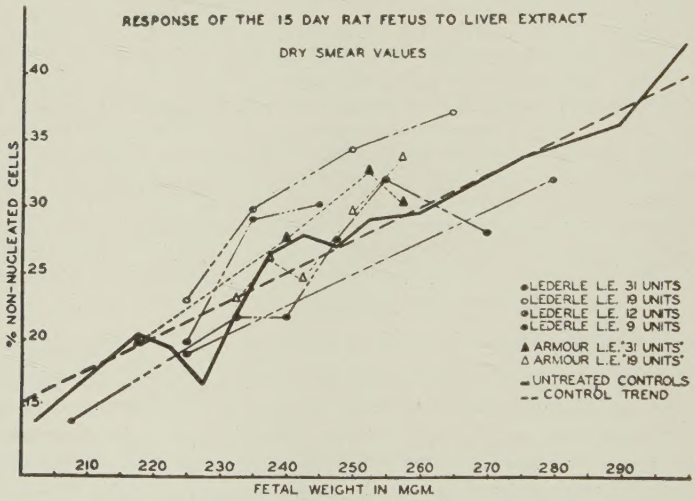

FIG. 1.

Is the response of the animals receiving liver therapy biologically significant? The distribution of variation of the control animals contains two components, *i. e.*, the variation at any given fetal weight and the upward trend. Since the trend factor is fortuitous and unnecessary for comparison of a treated and untreated animal at any given fetal weight, it can be eliminated. By fitting a straight line to the control curve by the least squares method and by calculating the distribution of variation of control values from the straight line, the standard deviation can be determined independently of the trend. The means of per cent counts on treated and untreated animals as compared with the means of the calculated normal response determined from the straight line equation are listed in

Table 3. In no instance does a mean of a treated group of rats exceed the calculated control value by the conventional two sigma.

TABLE 3.—COMPARISON OF DIFFERENTIAL COUNTS ON TREATED AND CONTROL 15-DAY RAT EMBRYOS.

Therapy.	Litters.	Fetuses.	Mean fetal weight (mg.).	Per cent non-nucleated cells (mean values). Wet smears. Expt.	Wet smears. Calc.†	Dry smears. Expt.	Dry smears. Calc.†
Controls (untreated)	30	120	240	22.4	22.4 $\sigma = 3.3$	26.2	26.3 $\sigma = 4.4$
Lederle liver extract:							
A. 9 units	3	12	240	19.6	22.2	18.7	25.8
B. 12 units	3	12	240	20.1	20.7	26.4	23.8
C. 19 units	6	24	240	25.9	22.1	30.0	25.4
D. 31 units	9	36	240	21.8	22.6	25.3	26.5
Armour liver extract:							
A. "19 units"*	8	32	240	26.4	22.6	26.5	25.4
B. "31 units"*	7	28	250	29.9	24.6	32.0	28.6

* A unit equivalent to 15 gm. of fresh beef liver.

† Means of values calculated from straight line equation fitted to control data by the least squares method where x = fetal weight and y = per cent non-nucleated cells.

To obviate the criticism that in a study of the 15-day embryo one would not observe the effects of the A-P-A principle on the primitive generation, it was necessary to investigate the effect of liver and Ventriculin on the immature primitive cells in the 11-day rat embryo. Table 4 summarizes the response of the 11-day embryo to anti-anemic therapy.

TABLE 4.—NUCLEAR DIAMETERS OF TREATED AND CONTROL 11-DAY RAT EMBRYOS

Therapy.	Litters.	Embryos.	Nuclear diameters (μ-average).
Controls (untreated)	12	50	8.5
Ventriculin*	7	28	8.4
Lederle liver extract:			
A. 12 units	4	19	8.6
B. 37 units	1	4	8.5
C. 45 units	1	4	8.5
D. 52 units	1	4	8.2
E. 60 units	4	16	8.3
F. 75 units	1	4	8.5

* Added as 2.5% of fox chow checquer diet.

In no cases is there a significant decrease in the nuclear diameters of a treated group of embryos over controls.

Discussion. The lack of a response of the opossum pouch-young and the rat embryo to the A-P-A principle necessitates a more critical evaluation of the fetal approach to the bio-assay problem. Since A-P-A factor is ineffective in altering the embryonic blood picture, either the embryo does not require the A-P-A principle, or it receives a sufficient supply from maternal stores, or it manufactures its own supply *de novo*. The assumption of placental transfer of the A-P-A factor though probable from indirect evidence is still open to question.

The fallacy in the rationale of the fetal approach is to disregard the past history of the erythrocytes in comparing the blood picture of the embryo with that of a pernicious anemia patient receiving

liver therapy. The macrocytes in the blood of pernicious anemia reflect a megaloblastic erythropoiesis which is an abnormal process. The large primitive erythrocytes in embryonic blood are the mature elements of primitive erythropoiesis which is a normal physiologic process. Furthermore, the immature cells of the primitive embryonic and megaloblastic generations are not cytologically identical.

It is difficult to reconcile the positive findings in the opossum pouch-young with those of the 11-day rat embryo. In the 11-day rat embryo, A-P-A factor might conceivably accelerate the intravascular maturation, of the immature primitive erythroblasts. In the 45-day opossum, the primitive generation has matured to the erythrocyte stage and is rapidly being replaced by small definitive red blood cells. Thus, any changes appearing in the peripheral blood must be due to the action of the A-P-A factor on the bone marrow or liver. No histologic evidence has been presented to indicate any stimulation of definitive erythropoiesis in opossum pouch-young or new-born rats. It has been conclusively demonstrated that the A-P-A factor does not affect definitive erythropoiesis in the normal animal. Furthermore, the response reported in opossum young is non-specific, in that gastric juice which is ineffective in the treatment of pernicious anemia also elicits a response in the opossum.

No explanation can be offered for the discrepancy between our findings and those in the literature on the opossum pouch-young and the 11-day rat embryo. The results of our experiments on the 20-, 15- and 11-day rat embryo and opossum pouch-young are in agreement with the findings of other workers who failed to detect any response of the normal mammalian embryo to anti-anemia therapy.

Summary. 1. Potent, standardized anti-anemia preparations were injected into or fed to pregnant rats and 45-day opossum pouch-young. Rats received therapy before, during, and after formation of the anlage of the mesoderm in the developing embryo.

2. No evidence of accelerated maturation as reflected by changes in the red cell count or mean corpuscular volume following liver therapy was observed in a study of 27 opossum pouch-young and 46 20-day rat embryos.

3. In a series of 264 treated and untreated 15-day fetuses from 66 litters of normal rats, statistical analysis of the data indicates the response in liver-treated animals to be within the range of biologic variation.

4. Liver and Ventriculin therapy did not alter the maturation rate of the primitive generation of red cells in 132 11-day rat embryos studied.

5. Embryogenesis of blood in the opossum and rat is unaffected by the antipernicious anemia principle and therefore neither opossum pouch-young nor rat embryos should be used to bio-assay anti-anemia preparations.

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ADDENDUM: After this manuscript was submitted for publication, a paper by O. P. Jones appeared (Arch. Int. Med., **68**, 476, 1941) questioning the lack of response of the 15- and 11-day embryos to anti-anemic therapy in the pregnant rat. The study of the 15-day rat embryo was criticized because "changes in the cells of the prehepatic generation are apt to be attenuated or completely masked by the rapidly growing normoblastic generation." This criticism, however, is apparently unsubstantiated by experimental facts. The circulating blood cells of the 15-day embryo are predominantly primitives of the prehepatic generation with relatively few definitives of the normoblastic generation. Since the maturation rate of both generations of embryonic red cells in the rat has been reported to be accelerated by anti-anemic therapy, the 15-day embryo should reflect changes in either or both generations of cells. Furthermore, hematopoietic responses in the 15-day embryo can be more easily and accurately determined in contrast with the tedious, impractical method of measuring the diameters of hundreds of cells in the 11-day embryo. However, no marked changes were observed in the blood pictures of either 15- or 11-day rat embryos after injection of potent anti-anemic preparations or the feeding of Ventriculin to pregnant rats.

As additional evidence that primitive prehepatic erythropoiesis can be altered by the anti-anemic principle, Jones quotes extensively the work of Sabin wherein the addition of liver extracts to chick blastoderms *in vitro* appeared to accelerate the maturation of the prehepatic embryonic red cells. Reference to the later work of Muller was omitted. Muller, with Sabin's assistance, failed to confirm Sabin's preliminary work on chick blastoderms.

The entire magnitude of the response to Ventriculin therapy in treated animals as reported by Jones was 5% or less. With a response of 1% to 5% it is necessary to evaluate critically the control line above which the response occurred. Jones' data are conspicuously lacking in adequate controls. In fact, it is difficult to ascertain what or where the controls are. No information as to the number of control animals is given. A textual reference to 5 rats is made with the statement, "This series could not be used in the present quantitative study because there were differences in time of mating the rats, their diets, and the method of sampling blood from the embryos." With no adequate control data the validity of 2% or 5% response based on data from 2 to 3 rats per dosage level is open to serious question. The value of such a response as a quantitative measure of the potency of anti-anemic preparations is questionable, especially when one considers that biologic variation in a large series of animals is conservatively estimated at 10% and that in most U.S.P. bio-assay methods a variation of ±20% of the standard is permissible.

Assuming that the "response" does occur, it cannot be concluded that the slight changes noted were due to the antipernicious anemia principle. The lack of adequate control experiments wherein other tissue extracts, prepared in a manner similar to Ventriculin concentrate, would be both injected and fed to pregnant rats makes it difficult to draw valid conclusions concerning specificity of Jones' observations.

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